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PRINCIPAL INVESTIGATOR: Wei Liu, M.D., Ph.D.
                        Javdutt Vadgama, Ph.D.

CONTRACTING ORGANIZATION: Charles R. Drew University
                          Los Angeles, California   90059

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6. AUTHOR(S)
Wei Liu, M.D., Ph.D.
Javdutt Vadgama, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Charles R. Drew University
Los Angeles, California 90059

E-MAIL:
weliu7@yahoo.com

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13. ABSTRACT (Maximum 200 Words)

IGF-I, a mitogen for human breast cancer, stimulates cell division by regulating events during the early G1 phase, but little is known about the signaling pathways involved. In this study, we examined the signal transduction pathways involved in IGF-I-induced cyclin D1 expression in MCF-7 human breast cancer cells. ERK1/ERK2 and JNK in MCF-7 cells were rapidly activated in response to IGF-I. Deprivation of PKC by PMA inhibited IGF-I-induced JNK activation, suggesting that IGF-I-induced JNK activation was PKC-dependent. However, PMA stimulated ERK1/ERK2 activation persistently in MCF-7 cells. IGF-I-induced ERK1/ERK2 and JNK activation could be blocked by staurosporine, a protein kinase C inhibitor, indicating that the activation of ERK1/ERK2 and of JNK by IGF-1 was associated with PKC pathway. IGF-I up-regulated both cyclin D1 mRNA and protein in MCF-7 cells. IGF-I-induced cyclin D1 mRNA and protein could be inhibited by two PI-3 kinase inhibitors, wortmannin and LY294002, instead of MAP kinase inhibitor. Our results suggest that 1). PKC pathway is involved in IGF-I-induced activation of ERK1/ERK2 and JNK in MCF-7 cells. 2). Our data confirm that IGF-I-induced cyclin D1 expression is mediated by PI-3 kinase pathway.

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FOREWORD

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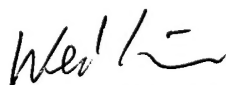
N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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Introduction

Cumulative evidence shows that insulin-like growth factor (IGF-I) plays an important role in the mitogenesis of breast cancer cells. IGF-I stimulates cell division by regulating events during the early G1 phase, but little is known about the signal transduction pathways involved in these events. The purposes for this proposal are: 1) what is the role of protein kinase C in the IGF-I-induced SAPK/JNK activation in human breast cancer cells MCF-7. 2). what is the relationship between IGF-I-induced SAPK/JNK signaling pathway and cyclic AMP-dependent protein kinase A signaling pathway. Our specific aims in this proposal are 1). To elucidate the role of PKC in IGF-I-mediated protooncogene c-jun phosphorylation in human breast cancer cells MCF-7. 2). To characterize the role of cyclic AMP in IGF-I-mediated protooncogene c-jun phosphorylation in MCF-7 cells. The significant achievement will be not only to gain further understanding how cellular activities are regulated in human breast cancer, but also because of the possibility that the information will be used to devise better therapies for the breast cancer.

I obtained a good training in molecular biology techniques under the guidance of my mentor Dr. Vadgama. For example, I learned Northern blot analysis technique in Dr. Vadgama's laboratory to detect the gene expression in mRNA level. Using Nothern blot analysis technique I learned I investigated cyclin D1 mRNA expression in human breast cancer cells MCF-7 in response to IGF-I. Also under Dr. Vadgama's guidance, I learned differential display of reverse transcription PCR (DDRT-PCR), one of the gene cloning techniques. I investigated genes differentially expressed in human breast cancer cells MCF-7 upon IGF-I stimulation or amino acid starvation using DDRT-PCR. I identified and characterized CD24 gene in MCF-7 cells upon amino acid starvation by means of DDRT-PCR technique I learned in Dr. Vadgama's laboratory.

Insulin-like growth factors (IGFs) have been shown to stimulate cell proliferation and differentiation. IGF-I, one of the important members of the IGF family plays an important role in the mitogenesis of breast cancer. IGF-I stimulates cell division by modulating events that occur during the early G1 phase. In the present study, we investigated signal transduction pathways involved in IGF-I-induced cyclin D1 expression. The phosphorylation of mitogen-activated protein kinase (ERK1/ERK2) and that of protooncogene c-jun in MCF-7 cells were rapidly activated in response to IGF-I. The stimulation was in a time/dose-dependent manner.

In order to study the role of protein kinase C on the activation of ERK1/ERK2 and SAPK/JNK in MCF-7 cells, cells were incubated with phorbol easter PMA which could down-regulate PKC activity. IGF-I-induced SAPK/JNK in MCF-7 cells was completely blocked through the down-regulation of PKC by PMA. However, ERK1/ERK2 in MCF-7 cells was persistently activated by the addition of PMA. Staurosporine, a PKC inhibitor could completely abolish IGF-I-induced ERK1/ERK2 and SAPK/JNK activation in MCF-7 cells. These data confirm that IGF-I-induced ERK1/ERK2 and SAPK/JNK activation in MCF-7 cells was PKC-dependent. PMA as a potential mitogen could persistently stimulate ERK1/ERK2 in MCF-7 cells indicating that persistent activation of ERK1/ERK2 may be essential for tumor cell proliferation. Cell proliferation and activation of oncogenes in cancer cells has been shown to be involved in the modulation of signal transduction pathways. The most important signal transduction pathways in mammalian cells are MAPK pathways, which include ERK1/ERK2, JNK and p38 pathways. These kinases are activated by growth factors and also under stress conditions. Our data demonstrated that there is cross-talk exert between MAPK and PKC signal transduction pathways. It may provide a new therapeutic method to treat breast cancer by interfere PKC pathway in breast cancer patients.

Since c-jun kinase is mainly activated under stress conditions. Amino acid starvation in cell lines can be used as an experimental model for stress, which can mimic the pathophysiological condition that results from protein deprivation during cancer cachexia. Therefore, we hypothesize that there are genes differentially expressed in MCF-7 cells in response to amino acid starvation. We identified and characterized CD24 gene differentially expressed in MCF-7 cells upon amino acid starvation.

Results: ERK1/ERK2 and JNK in human breast cancer cells MCF-7 were rapidly activated in response to the addition of IGF-I (**Figure 1**). Staurosporine, a PKC inhibitor could block IGF-I-induced ERK1/ERK2 and JNK activation (**Figure 2**). We have shown that IGF-I stimulates cyclin D1 expression in MCF-7 cells in a time and dose-dependent manner (**Figure 3**). The maximum stimulation was observed at 18 to 24 hours in the 10 ng/ml of IGF-I concentration. Cyclin D1 mRNA was blocked by the addition of PI-3 kinase inhibitors (**Figure 4**). Our studies suggest that cyclin D1 expression is regulated by IGF-I at the transcription level and that the phosphorylation pathways are regulated by PI-3 kinase signaling pathway in addition to MAPK pathways.

The effect of amino acid starvation on the regulation of CD24 gene in MCF-7 cells was investigated. An increase in CD24 mRNA expression was observed in a time-dependent manner (**Figure 5**) with peak stimulation after 8 h amino acid withdrawal. This up-regulation of CD24 mRNA by amino acid starvation occurred at transcriptional level (**Figure 6**).

Publications:

1. Liu W. and Vadgama JV. Identification and characterization of amino acid starvation-induced CD24 gene in MCF-7 human breast cancer cells. *Intl. J. Oncol* 16:1049-1054, 2000.

2. Liu W, Chillar, and Vadgama JV. IGF-I-induced mitogenesis of breast cancer cells involves in MAPK and the PI-3 kinase pathways. *Pro. Am. Assoc. Cac.* Res. (39):458, 1998.

3. Liu W, Vadgama JV, and Wu Y. Signal transduction pathways in breast cancer cell MCF-7 upon amino acid starvation involved in MAPK and SAPK/JNK pathways. *FASEB J.* (12):A450, 1998.

cc. Mentor: JV. Vadgama, Ph. D.
cc. Grant Office of Charles R. Drew University of Medicine and Science

Key Research Accomplishments

1. Elucidated that IGF-I-induced ERK1/ERK2 and SAPK/JNK activation in human breast cancer cells is PKC-dependent.

2. Demonstrated that PI-3 kinase pathway is involved in IGF-I-mediated cyclin D1 expression in MCF-7 cells.

3. Amino acid starvation regulates CD24 mRNA expression in MCF-7 cells and this regulation occurred at transcriptional level.

Reportable outcomes:

IGF-I-induced ERK1/ERK2 and SAPK/JNK activation in human breast cancer cells is PKC-dependent.

PI-3 kinase pathway is involved in IGF-I-mediated cyclin D1 expression in MCF-7 cells instead of MAPK pathway.
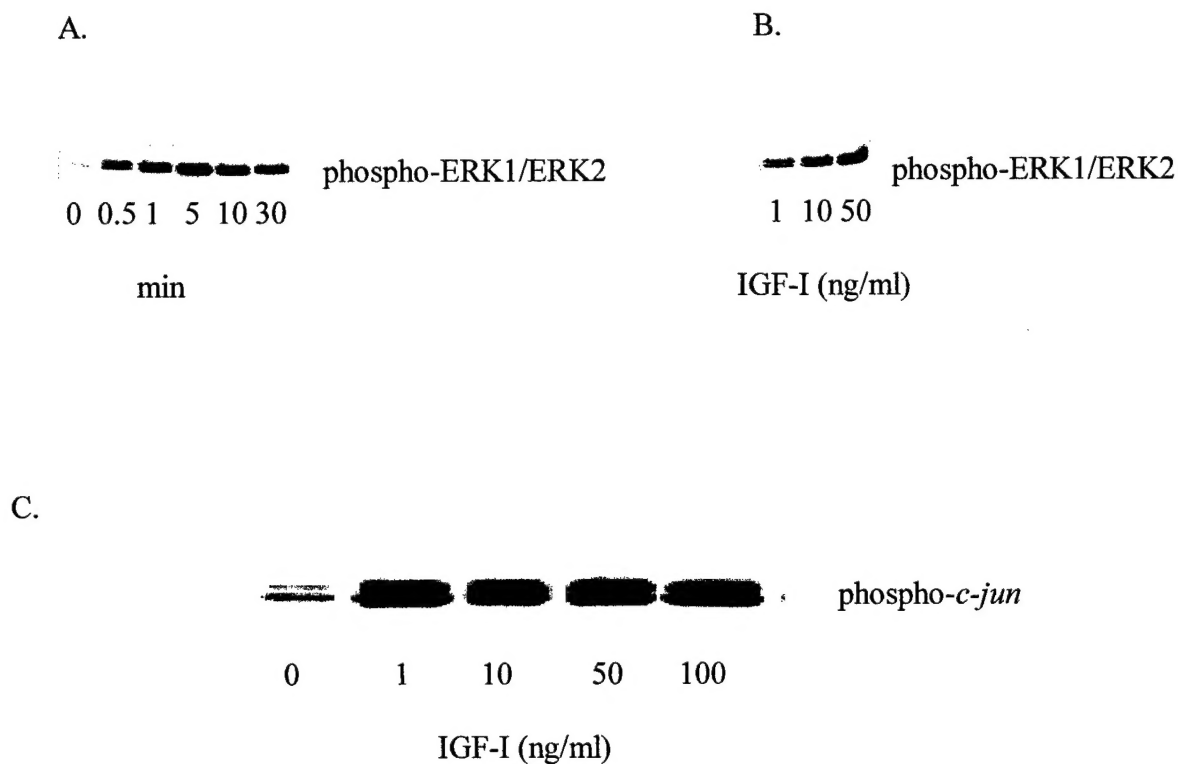
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Liu W. and Vadgama JV. Identification and characterization of amino acid starvation-induced CD24 gene in MCF-7 human breast cancer cells. *Intl. J. Oncol* 16:1049-1054, 2000.

Appendix 1.

Fig. 1

IGF-I-induced the activation of ERK1/ERK2 and JNK in MCF-7 cells

A.

 phospho-ERK1/ERK2

0 0.5 1 5 10 30

min

B.

 phospho-ERK1/ERK2

1 10 50

IGF-I (ng/ml)

C.

 phospho-*c-jun*

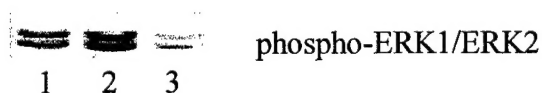0   1   10   50   100

IGF-I (ng/ml)

Legend:

MCF-7 cells were synchronized in RPMI-1640 serum-free medium over night. After synchronization, cells were treated with 10 ng/ml IGF-I for indicated time (A), various concentrations of IGF-I were added to the cells for 5 min (B), and cell lysates were collected. Western blot analyses were performed using phospho-ERK1/ERK2 antibody as a probe. In vitro kinase reaction was performed (C) and Western blot analyses were performed using phospho-c-jun antibody as a probe.

Fig. 2

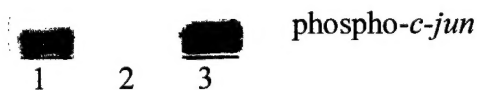**Staurasporine, a PKC inhibitor inhibits IGF-I-induced activation of ERK1/ERK2**

and JNK in MCF-7 cells

A.

phospho-ERK1/ERK2

1   2   3

1. control
2. IGF-I
3. 1 µM staurosporine + IGF-I

B.

phospho-*c-jun*

1   2   3

1. control
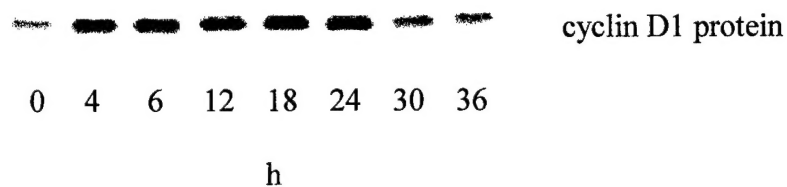2. 1 µM staurosporine + IGF-I
3. IGF-I

Legend:

MCF-7 cells were synchronized in RPMI-1640 serum-free medium over night. After synchronization, cells were treated with RPMI-1640 medium only (control), 10 ng/ml IGF-I (IGF-I), or 1 µM staurosporine plus IGF-I (staurosporine + IGF-I) for 5 min. Cell lysates were collected and in vitro kinase assay was performed (B). Western blot analyses were performed using antibodies phospho-ERK1/ERK2 (A) and phospho-c-jun (B).
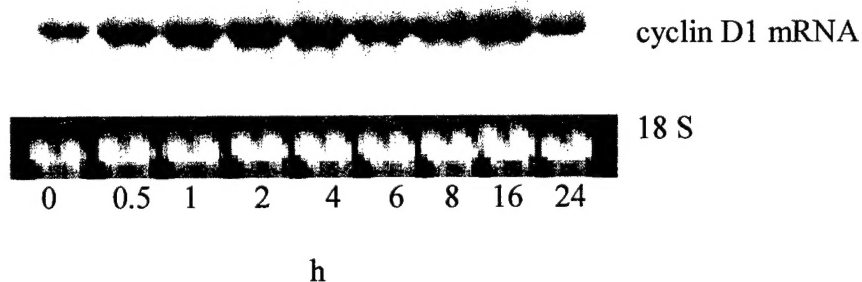
Appendix 3.

Fig. 3

IGF-I stimulates cyclin D1 expression in MCF-7 cells

A.

 cyclin D1 protein

0   4   6   12   18   24   30   36

h

B.

 cyclin D1 mRNA

 18 S

0   0.5   1   2   4   6   8   16   24

h

C.

 cyclin D1 protein

0   0.1   1   10   50   100

IGF-I (ng/mg)

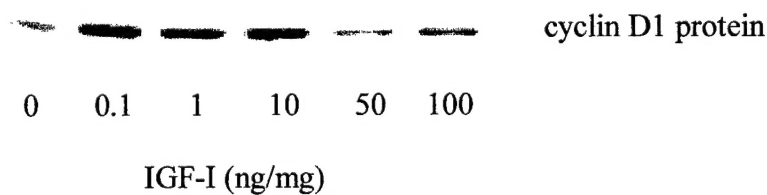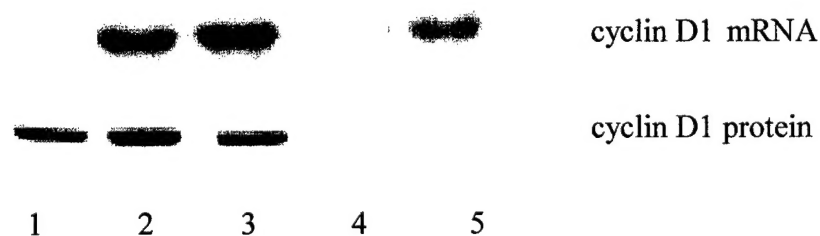**Legend:**

MCF-7 cells were synchronized in RPMI-1640 serum-free medium over night. Cells were then treated with 10 ng/ml IGF-I for indicated times (A, B) and various concentrations of IGF-I were added into culture for 18 h (C). Cell lysates were collected (A, C) and cellular RNA (B) was isolated. Western blot analyses were performed (A, C) using human cyclin D1 antibody. Northern blot analysis was performed using human cyclin D1 cDNA probe.

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Appendix 4.

Fig. 4

The roles of MAPK and PI-3 kinase pathways on
IGF-I-induced cyclin D1 mRNA and protein expression in MCF-7 cells

cyclin D1 mRNA

cyclin D1 protein

1   2   3   4   5

1. synchronized only
2. 10 ng/ml IGF-I
3. 20 µM of PD98059 + IGF-I
4. 10 µM of wortmannin + IGF-I
5. 100 µM of LY294002 + IGF-I

Legend:

MCF-7 cells were maintained in RPMI-1640 medium. After over night synchronization, cells were treated with various reagents as indicated for 18 h (for protein analysis) or for 6 h (for mRNA analysis). Cell lysates and cellular RNA were collected. Western blot analysis using human cyclin D1 antibody and Northern blot analysis using human cyclin D1 cDNA as a probe were performed.

Expression of CD24 mRNA in MCF-7 cells in response to amino acid starvation


```
              - CD24

              - GAPDH

0  1  3  8  16  24  48  C

          h (-AA)
```
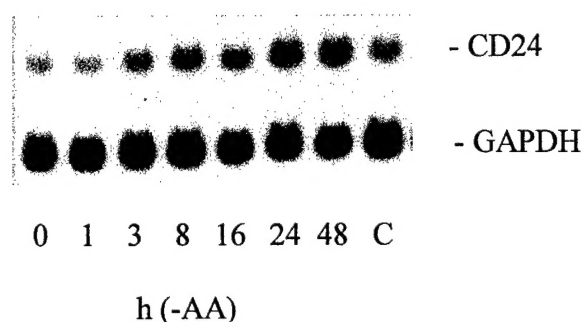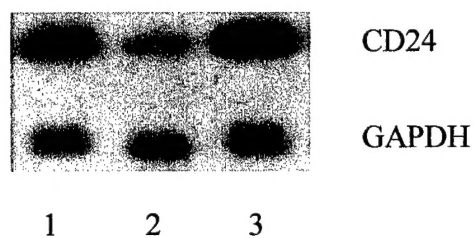
Legend:

Expression of CD24 mRNA in MCF-7 cells in response to amino acid starvation. MCF-7 cells were cultured in RPMI-1640 medium. When the cells reached to 95% confluence, the medium was removed and RPMI-1640 serum-free medium was added over night. After overnight synchronization the cells were incubated with HBSS plus 5% of DFBS (-AA) for indicated time. Cellular RNA was isolated and Northern blot analysis was performed using human CD24 cDNA probe derived from DDRT-PCR. In order to exclude the effect of DFBS on the induction of CD24 mRNA expression, cells were incubated with RPMI-1640 medium containing 5% of DFBS for 24 h (Lane C).

Appendix 6.

Fig. 6

Amino acid starvation-induced CD24 mRNA expression in MCF-7 cells is regulated at transcriptional level

Legend:

Amino acid starvation-induced CD24 mRNA expression in MCF-7 cells is regulated at transcriptional level. MCF-7 cells were maintained in RPMI-1640 medium containing 10% of FBS. When cells reached to 95% confluence, cells were incubated with HBSS plus 5% DFBS (lane 1), 20 µg/ml of actinomycin D (lane 2) and 10 µg/ml of cycloheximide (lane 3) for 24 h. Cellular RNA was isolated and Northern blot analysis was performed using human CD24 cDNA probe derived from DDRT-PCR.